Multifunctional RNA silencing pathway polymerase QDE-1 of *Neurospora crassa*

Antti Aalto

Institute of Biotechnology and
Department of Biosciences
Division of General Microbiology
Faculty of Biological and Environmental Sciences
and
Helsinki Graduate Program in
Biotechnology and Molecular Biology
University of Helsinki

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Supervisors

Professor Dennis Bamford
Institute of Biotechnology and Department of Biosciences
University of Helsinki
Finland

Docent Minna Poranen
Institute of Biotechnology and Department of Biosciences
University of Helsinki
Finland

Reviewers

Docent Mikko Frilander
Institute of Biotechnology
University of Helsinki
Finland

Docent Sakari Kauppinen
Copenhagen Institute of Technology
Aalborg University
Denmark

Opponent

Professor Garry Wong
A. I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
Finland

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ABBREVIATIONS

AGO	Argonaute
ARC	Argonaute siRNA chaperone
aRNA	aberrant RNA
ATP	adenosine-5'-triphosphate
Aub	Aubergine
BLM	Bloom syndrome
bp	base pair
C3PO	component 3 promoter of RISC
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CTP	cytidine-5'-triphosphate
DCL	Dicer-like
Dcr	Dicer
DdRP	DNA-dependent RNA polymerase
DGCR8	DiGeorge syndrome critical region gene 8
disiRNA	Dicer-independent small interfering RNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPBB	double-psi β-barrel
DRAG	dsRNA-activated gene
ds	double-stranded
GTP	guanosine-5'-triphosphate
His	histidine
Loqs	Loquacious
mat	mating type
MID	middle domain of Argonaute
miRNA	micro RNA
milRNA	miRNA-like small RNA
mRNA	messenger RNA
MSUD	meiotic silencing of unpaired DNA
NOR	nucleolus organizer region
nt	nucleotide
NTP	nucleoside triphosphate
ORF	open reading frame
Pasha	partner of Drosha
PAZ	Piwi-Argonaute-Zwille
P-body	processing body
PCR	polymerase chain reaction
piRNA	Piwi-interacting RNA
PIWI	P-element induced wimpy testis
Pol	polymerase
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
qa	quinic acid
QDE	quelling defective
QIP	QDE-2-interacting protein
qiRNA	QDE-2-interacting small RNA
qRT-PCR	quantitative real-time PCR
rDNA	ribosomal DNA
RDR	RNA-dependent RNA polymerase
RDRC	RNA-dependent RNA polymerase complex
RdRP	RNA-dependent RNA polymerase
RIP	repeat-induced point mutation
RISC	RNA-induced silencing complex
RITS	RNA-induced initiation of transcriptional gene silencing
RLC	RISC-loading complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RPA	Replication Protein A
rRNA	ribosomal RNA
scnRNA	scan RNA
siRNA	small interfering RNA
TNTase	terminal nucleotidyl transferase
tRNA	transfer RNA
UTP	uridine-5'-triphosphate
UTR	untranslated region
WRN	Werner syndrome
wt	wild type
Å	ångström

SUMMARY

Double-stranded RNA and associated proteins are known to regulate the gene expression of most eukaryotic organisms. These regulation pathways have different components, outcomes and distinct nomenclature depending on the model system, and often they are referred to collectively as RNA silencing. In many cases, RNA-dependent RNA polymerases (RdRPs) are found to be involved in RNA silencing, but their targets, activities, interaction partners and reaction products remain enigmatic. In the filamentous fungus *Neurospora crassa*, the RdRP QDE-1 is critical for silencing of transgenes – a phenomenon known as quelling.

In this thesis the structure, biochemical activities and biological functions of QDE-1 were extensively studied. This dimeric RdRP was shown to possess five distinct catalytic *in vitro* activities that could be dissected by mutagenesis and by altering reaction conditions. The biochemical characterization implied that QDE-1 is actually an active DNA-dependent RNA polymerase that has additional RdRP activity. It also provided a structural explanation for the dimerization and suggested a biological framework for the functions of QDE-1 *in vivo*. (**I**)

QDE-1 was also studied in a broader context along with the other components of the quelling pathway. It was shown that DNA damage in *Neurospora* causes a dramatic increase in the expression level of the Argonaute protein QDE-2 as well as the synthesis of a novel class of small RNAs known as qiRNAs. The accumulation of qiRNAs was shown to be dependent on several quelling components, and particularly to be derived from an aberrant ssRNA (aRNA) molecule that is synthesized by QDE-1 in the nucleus. The genomic distribution of qiRNA targets was analyzed and the possible biological significance of qiRNAs was studied. Importantly, qiRNAs are the first class of small RNAs that are induced by DNA damage. (**II**)

After establishing that QDE-1 is a multifunctional RNA polymerase with several activities, template specificities and subcellular locations, the focus was turned onto its interaction partners. It had been previously known that QDE-1 associates with Replication Protein A (RPA), but the RecQ helicase QDE-3 was now shown to regulate this interaction. RPA was also observed to promote QDE-1 dependent dsRNA synthesis *in vitro*. By characterizing the interplay between QDE-1, QDE-3 and RPA, a working model of quelling and qiRNA pathways in *Neurospora* was presented. (**III**)

This work sheds light on the complexity of the various RNA silencing pathways of a fungal model system. It shows how an RdRP can regulate gene expression on many levels, and suggests novel lines of research in other eukaryotic organisms.

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1 INTRODUCTION

1.1 RNA Silencing

1.1.1 Discovery of RNA silencing

RNA is a critical catalytic component in diverse biochemical events ranging from intron splicing and protein translation to telomere function (Cech, 2009; Korostelev & Noller, 2007). In addition to its role in enzymatic catalysis, recent developments in molecular biology have brought about an appreciation of the importance of RNA in gene expression regulation. Double-stranded RNA (dsRNA) in particular has been implicated in many cellular pathways as a crucial regulatory molecule (Bartel, 2004; Moazed, 2009; Siomi & Siomi, 2009). RNA silencing is a term encompassing all the various sequence-specific effects that dsRNA exerts in eukaryotic cells (Meister & Tuschl, 2004).

The discovery of RNA silencing is often attributed to one seminal study carried out in the worm *Caenorhabditis elegans* (Fire et al, 1998). However, implications of unusual regulatory events were detected already several years earlier by a research group studying petunia plants (Napoli et al, 1990). Chalcone synthase (CSH) is a critical enzyme in the biosynthetic pathway of flavonoids that dictate the coloration of flowers. When the authors introduced a transgenic form of the CSH gene in petunias, they observed flower color phenotypes that were different from the parental plants. Expression of the transgenic gene caused a dramatic reduction in the levels of the endogenous CSH gene expression. This phenomenon was assigned as "co-suppression". The mechanism underlying these observations was not understood, but it was hypothesized that it might be associated with DNA methylation (Napoli et al, 1990).

Two years later, a group studying the filamentous fungus *Neurospora crassa* made similar observations (Romano & Macino, 1992). They transformed wild-type *Neurospora* strains with different portions of two genes important for carotenoid biosynthesis (*albino-1* and *albino-3*), and observed that the transformants displayed white and yellow phenotypes differing from the bright orange color of the wild-type. They showed that the mutant phenotypes result from transcription impairment of the endogenous genes. The mutants tended to revert to the wild-type phenotype and this reversion was correlated with the loss of exogenous gene copies. This phenomenon was named "quelling" and a few years later it was shown not to involve DNA methylation but a diffusible, trans-acting signal operating at the post-transcriptional level (Cogoni et al, 1996).

The revolution of regulatory dsRNA began by the discovery of RNA interference (RNAi) by Andrew Fire, Craig Mello and co-workers (Fire et al, 1998; Rocheleau et al, 1997). At this time, many researchers were attempting to specifically knock down genes by so-called antisense strategies where either oligonucleotides or bioengineered ribozymes complementary to target messenger RNAs (mRNAs) were introduced in cells (Branch, 1998; Fire et al, 1991). The results were often variable or non-specific, and the control sense strand sometimes gave identical results to the antisense strand (Guo & Kemphues, 1995). When developing the antisense methodology for the myofilament protein UNC-22 of C. elegans, Fire and co-workers noticed that mixtures of *unc-22* sense and

antisense RNA strands were much more potent in silencing the expression of the target mRNA than either of the single strands alone (Fire et al, 1998). They further showed that dsRNA was a very effective silencing inducer and confirmed the sequence-specificity of silencing by several distinct targets. In addition, the mutant phenotypes were detectable in the progeny of the dsRNA-treated animals, and the silencing was able to spread across cellular boundaries from the dsRNA injection site. For these discoveries, Fire and Mello were awarded the 2006 Nobel Prize in Physiology or Medicine.

1.1.2 Components of RNA silencing pathways

1.1.2.1 Double-stranded RNA

Soon after the first description of the RNAi phenomenon, short RNA species of approximately 21 to 25 nucleotides (nts) in length were discovered that were suggested to directly guide mRNA cleavage (Hamilton & Baulcombe, 1999; Hammond et al, 2000; Zamore et al, 2000). These were subsequently named short interfering RNAs (siRNAs) and shown to be processed from long dsRNA by an RNase III-like mechanism (Elbashir et al, 2001b). A class of endogenous small non-coding RNAs (micro RNAs or miRNAs) that regulate the expression of cognate genes in *C. elegans* and other organisms was also described (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee & Ambros, 2001). The discovery of repeat-associated siRNAs (now known as Piwi-interacting RNAs or piRNAs) (Aravin et al, 2001) established the three major groups of small regulatory RNA species known today. There are, however, several minor groups of small RNAs that do not exactly fit to these categories and have been given distinct names (Ghildiyal & Zamore, 2009).

The presence of dsRNA in eukaryotic cells has traditionally been regarded as an indication of a virus infection. In mammalian cells there are two main pathways that are induced by interferons and activated by dsRNA (Stark et al, 1998). The RNA-regulated protein kinase R (PKR) is a serine-threonine kinase that is normally inactive in cells. Upon activation by dsRNA it undergoes autophosphorylation and phosphorylates the α-subunit of the protein synthesis initiation factor eIF2, which leads to inhibition of translation (Clemens, 1997; Hovanessian, 2007). On the other hand, dsRNA also activates the 2',5'-oligoadenylate synthetase (2',5'-OAS) which catalyzes the conversion of ATPs to oligoadenylates linked by a 2',5'-bond instead of the normal 3',5'-phosphodiester bond. These molecules activate the 2',5'-OA-dependent RNase L which leads to extensive non-specific degradation of single-stranded RNA (ssRNA) (Hovanessian, 2007; Stark et al, 1998). The activation of these pathways eventually leads to apoptosis.

However, eukaryotic cells contain a number of sources for "natural" dsRNA. Most organisms contain RNA-dependent RNA polymerases (RdRPs) that are capable of converting ssRNA into dsRNA (see below). dsRNA may also be formed through normal transcriptional events. For example, the L1 retrotransposon contains both sense and antisense promoters and could promote bidirectional transcription of complementary ssRNA molecules (Ghildiyal & Zamore, 2009). Also closely related genes and pseudogenes in inverted orientations, as well as transcripts having stem-loop structures might be sufficient for dsRNA formation and subsequent regulatory events (Tam et al, 2008; Watanabe et al, 2008). The interferon

response in experimental work with dsRNA is yet an unresolved issue. In many cases the apoptotic dsRNA response can be circumvented (Elbashir et al, 2001a), while in other instances it clearly is a problem (Bridge et al, 2003; Sledz et al, 2003).

A general description of various RNA silencing mechanisms is shown in **Figure 1**. siRNAs are derived from long dsRNA through the action of the RNase III enzyme Dicer. They associate with Argonaute proteins, become single-stranded and guide the sequence-specific degradation of cognate mRNAs. Micro RNAs on the other hand are encoded by miRNA genes and processed in the nucleus by another RNase III enzyme Drosha (Lee et al, 2003). miRNAs are further cleaved in the cytoplasm by Dicer, and upon association with Argonautes they usually lead to translational inhibition. The piRNA pathway functions primarily in the germline of animals and is independent of RNase III enzymes. The specifics of these pathways are described below.

1.1.2.2 Argonaute proteins

Argonaute (AGO) proteins are essential components of the effector complexes that link the small RNAs to cellular functions (Hock & Meister, 2008; Hutvagner & Simard, 2008). First identified in the plant *Arabidopsis thaliana* (Bohmert et al, 1998), AGOs are defined by the presence of four characteristic domains: N-terminal, PAZ (Piwi-Argonaute-Zwille), MID (Middle) and PIWI (P-element induced wimpy testis) (Jinek & Doudna, 2009). Argonaute genes are conserved among all eukaryotes and their numbers range from just one in *Schizosaccharomyces pombe* to some 27 in *C. elegans* (Hock & Meister, 2008). Very soon after their discovery AGOs were implicated in RNA silencing (Grishok et al, 2001; Tabara et al, 1999) and shown to be required for the assembly of RNA-induced silencing complexes (RISCs) (Hammond et al, 2000; Hammond et al, 2001). An activated RISC is a complex where a single-stranded small regulatory RNA guides the AGO protein to exert its sequence-specific effect to an RNA target (see **Figure 1**).

Solving the crystal structure of an archaeal Argonaute protein from *Pyrococcus furiosus* shed some light on the functions of the different AGO domains (Song et al, 2004). The N-terminal, MID and PIWI domains form a crescent-shaped platform above which the PAZ domain arches through a stalk connecting the N-terminal and PAZ domains. The PAZ domain contains a binding pocket that is able to bind ssRNA. Interestingly, the double-stranded cleavage products of Dicer contain two nucleotides long single-stranded 3'-overhangs (see **1.1.2.3**). The pocket of the PAZ domain fits these overhangs snugly (Lingel et al, 2004).

The structure of the PIWI domain was, however, even more striking and suggested an immediate function for Argonautes (Song et al, 2004). The PIWI domain adopts a fold of RNase H, which is known to cleave RNA on DNA/RNA hybrids (Hausen & Stein, 1970). The RNA product of an RNase H catalyzed cleavage reaction contains a 3' hydroxyl and a 5' phosphomonoester, which can also be found at the ends of a target mRNA after the RISC catalysis (Martinez & Tuschl, 2004). Also, mRNA cleavage by RISC is endonucleolytic and dependent on Mg^{2+} ions (Schwarz et al, 2004). These findings suggested a model for mRNA cleavage by RISC where the 3' end of the single-stranded siRNA binds to the PAZ domain of the AGO protein, guiding the mRNA in the vicinity of the RNase H-like PIWI domain by base-pairing. This interaction would place the scissile bond of the mRNA between nucleotides 11 and 12 counting from the 3´end of the guide siRNA, which correlated with previous findings (Elbashir et al,

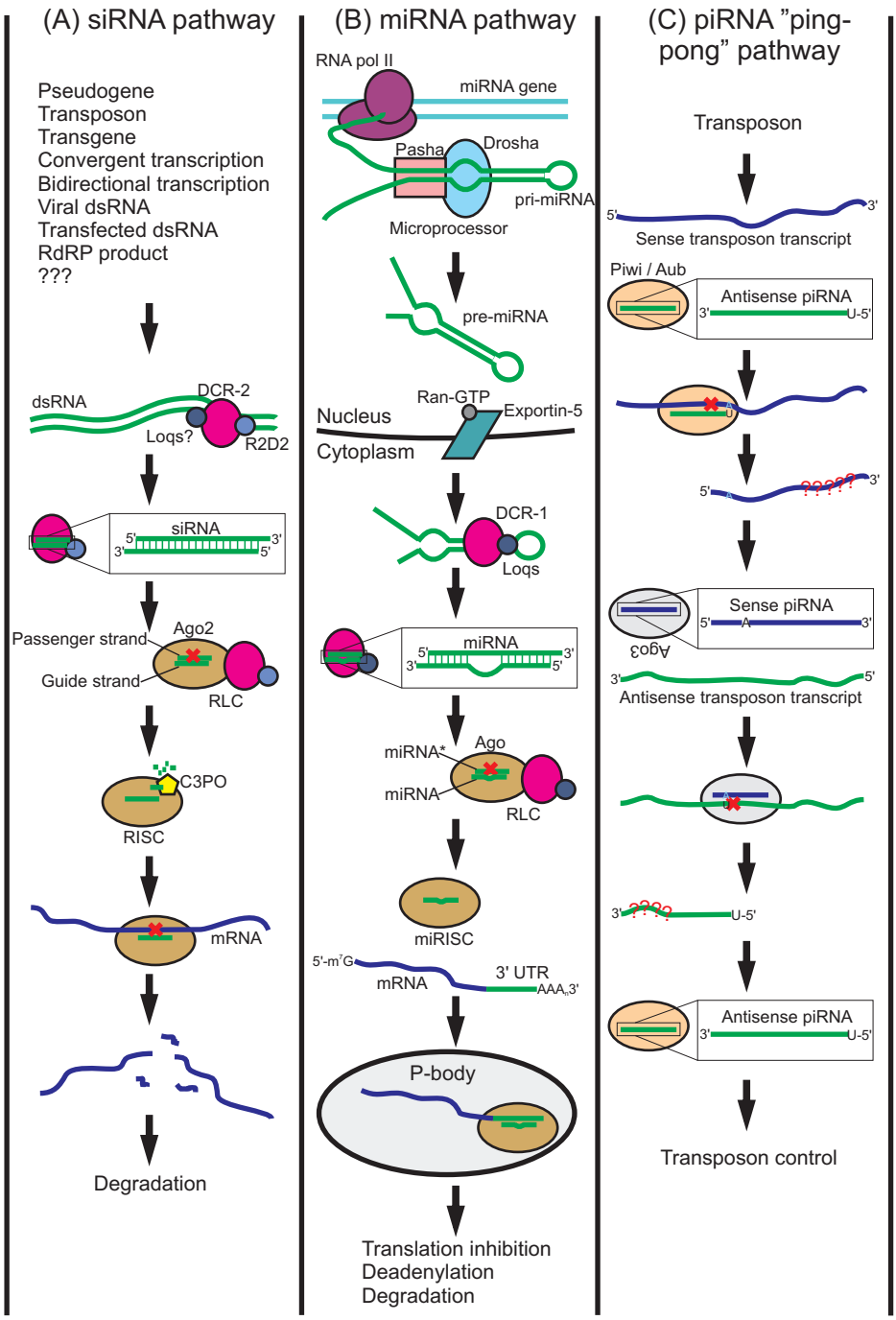


Figure 1. RNA silencing pathways.
A schematic representation of the most common RNA silencing pathways. The nomenclature of the components is as they are known in *Drosophila*. The picture was made according to Ghildiyal & Zamore, 2009 and Brennecke et al, 2007. See text for details.

2001b; Martinez & Tuschl, 2004) and strengthened the idea that the "slicer" activity of RISC resides within the Argonaute. This was confirmed by an elegant study showing that the mammalian Ago2, bound to a cognate siRNA, is capable of sequence-specific cleavage of a target mRNA (Liu et al, 2004). The slicer activity of AGOs was later described in other organisms as well (Baumberger & Baulcombe, 2005; Miyoshi et al, 2005). The molecular architectures of full-length eukaryotic Argonautes are not yet known, but recently the crystal structures of human AGO2 and *Neurospora* QDE-2 MID domains were solved (Boland et al, 2010; Frank et al, 2010). MID domains of AGO proteins are known to be involved in the binding of the 5' ends of guide RNAs (Jinek & Doudna, 2009). The structure of the AGO2 MID domain revealed a loop that makes specific contacts with AMP and UMP, but discriminates against GMP and CMP. This was suggested to be one of the mechanistic explanations for the 5' nucleotide bias often observed in miRNAs and other small RNAs (Frank et al, 2010). The QDE-2 MID domain structure revealed a potential second ligand binding pocket in addition to the pocket binding to the 5' nucleotide of the guide strand. The function of this second binding site remained elusive, but it was suggested to be involved in allosteric regulation (Boland et al, 2010).

Currently, Argonautes are divided in three categories or clades: Argonaute-like (Ago), Piwi-like and group 3 Argonautes, which have so far been found only in *C. elegans* (Hutvagner & Simard, 2008). In mammals there are in total eight Argonaute genes, four of which belong to the Ago-clade (Ago1-4) and four to the Piwi-clade (HIWI1-3, HILI) (Peters & Meister, 2007). Although all the Ago-proteins are highly homologous, only Ago2 is has endonucleolytic activity (Liu et al, 2004; Meister et al, 2004). Members of the Piwi-clade are regarded to be restricted to the germline and associate exclusively with piRNAs (Hock & Meister, 2008). Very recent studies suggest, however, that the Piwi proteins would have functions in somatic cells as well (Li et al, 2009; Malone et al, 2009).

1.1.2.3 Dicer and Drosha

Dicer and Drosha are RNase III enzymes that are an intrinsic part of siRNA and miRNA silencing pathways (Bernstein et al, 2001; Lee et al, 2003; Wu et al, 2000). Both specifically cleave dsRNA, but have different substrate specificities. In the nucleus, Drosha processes primary miRNA transcripts (pri-miRNAs) into precursor miRNAs (pre-miRNAs) within the Microprocessor complex (Denli et al, 2004; Gregory et al, 2004). Dicer on the other hand produces both siRNAs and miRNAs in the cytoplasm.

As with AGO encoding genes, the numbers of Dicer genes vary between different organisms. Mammals and *C. elegans* have only one Dicer, but *Drosophila melanogaster* and *Neurospora* have two (Catalanotto et al, 2004; Ghildiyal & Zamore, 2009). The reaction products of RNase III enzymes have characteristic 5' monophosphates and two-nucleotide single-stranded 3' overhangs at their ends. Human Dicer and Drosha contain two tandemly arranged RNase III domains as well as a dsRNA binding domain (dsRBD). In addition, Dicer contains the ssRNA-binding PAZ domain, already familiar from the Argonaute proteins (Jinek & Doudna, 2009).

The crystal structure of Drosha has not yet been determined, but the structure of *Giardia intestinalis* Dicer was solved at 3.3 Å resolution (Macrae et al, 2006). This structure resembles that of an axe, where the two RNase III domains (RNase IIIa and RNase IIIb) come together as an intramolecular dimer to form the blade and the PAZ domain forms the handle. In a bacterial RNase III two separate

polypeptide chains form an intermolecular dimer that has a similar fold (Gan et al, 2006). Each of these subunits catalyzes a metal-coordinated cleavage of one strand of the dsRNA duplex. In the *Giardia* Dicer the RNase IIIa and RNase IIIb domains each direct a two-metal-ion catalyzed endonucleolytic cleavage of RNA, giving rise to the characteristic termini observed in siRNA and miRNA molecules. The Dicer itself acts as a molecular ruler as the 3' end of the substrate dsRNA binds to the PAZ domain, placing the scissile bonds exactly 25 bp away from the 3' end (Macrae et al, 2006). If the PAZ domain is deleted, the resulting RNA cleavage products are of variable sizes (MacRae et al, 2007). The additional domains found in other Dicers probably have important functions in association with other proteins and regulatory events (Jinek & Doudna, 2009).

Dicer and Drosha do not work alone. Drosha forms the Microprocessor complex together with a protein called Pasha (partner of Drosha) in *Drosophila* and DGCR8 in humans (Denli et al, 2004). It has been suggested that DGCR8 interacts directly with the pri-miRNA transcript and functions as a molecular anchor to guide Drosha cleavage (Han et al, 2006). Dicers are known to associate with dsRNA-binding proteins called R2D2 and Loquacious (Loqs, TRBP in humans) (Chendrimada et al, 2005; Forstemann et al, 2005; Liu et al, 2003). In *Drosophila*, Dcr-1 usually interacts with Loqs and Dcr-2 with R2D2, bringing specificity to miRNA and siRNA pathways (see **Figure 1**). As R2D2 and Loqs are required for efficient Dicer cleavage and subsequent RISC function, they are said to form RISC loading complexes (RLCs) with Dicers (Ghildiyal & Zamore, 2009).

1.1.2.4 RNA-dependent RNA polymerases

RNA-dependent RNA polymerases (RdRPs) can be classified into those of viral and cellular origin. It has been shown that although these enzymes catalyze similar reactions, they are structurally and evolutionarily distinct (Iyer et al, 2003; Salgado et al, 2006). Viral RdRPs have been extensively studied and are reviewed elsewhere (Makeyev & Grimes, 2004; van Dijk et al, 2004). The following discussion concentrates exclusively on cellular, eukaryotic RdRPs.

The first cellular RdRP free from contaminating activities was purified from tomato leaves (Schiebel et al, 1993a; Schiebel et al, 1993b; Schiebel et al, 1998). It was shown that this polymerase is capable of both primed and unprimed (*de novo*) RNA synthesis on short RNA and DNA templates, and that homologous RdRP sequences are present in the genomes of other plants, yeasts and nematodes. RNA silencing in *Neurospora* was subsequently shown to be dependent on an RdRP (Cogoni & Macino, 1999a), and shortly after this the same was observed in *C. elegans* (Smardon et al, 2000). Today, database searches have revealed 161 putative genes encoding for RdRPs in 56 species of eukaryotes (Zong et al, 2009). For example, *C. elegans* has four RdRP genes while *Arabidopsis* has six. Out of the three RdRPs of *Neurospora* only QDE-1 is implicated in RNA silencing (Forrest et al, 2004). Interestingly, no RdRP homologs have been found in insect or vertebrate genomes.

The most studied eukaryotic RdRPs are QDE-1 of *Neurospora* and RDR6 of *Arabidopsis* (Curaba & Chen, 2008; Makeyev & Bamford, 2002; Salgado et al, 2006). Both are implicated in RNA silencing pathways and are capable of primer-independent RNA synthesis on an ssRNA template. In addition, RDR6 has been shown to possess a terminal nucleotidyltransferase (TNTase) activity as well as to be capable of using ssDNA as its template for RNA synthesis (Curaba &

Chen, 2008). It is unable to distinguish between 5'-capped and uncapped RNA templates, or to recognize the presence or absence of a poly(A) tail. QDE-1 is the only eukaryotic RdRP whose crystal structure has been solved (Salgado et al, 2006). Its biochemical and structural properties are described extensively in chapter **1.3.2.1**.

There are a few other direct examples of biological functions for eukaryotic RdRPs. Rdp1 of *S. pombe* has been shown to be essential for heterochromatin formation and maintenance (Sugiyama et al, 2005). Secondary siRNAs form a major class of small RNAs in *C. elegans* and each is a product of an RdRP synthesis event (Aoki et al, 2007; Pak & Fire, 2007; Sijen et al, 2007). In *Tetrahymena termophila*, RdRP Rdr1 and Dicer Dcr2 form a complex called RDRC that produces small RNA duplexes of 23 to 24 nucleotides (Lee & Collins, 2007). Recently, RdRP activities have been discovered also in insects and vertebrates. In *Drosophila*, a subunit of the RNA polymerase II elongator complex called D-elp1 was shown to have robust RdRP activity (Lipardi & Paterson, 2009). It also interacts with Dcr-2 and is associated with transposon suppression. Perhaps even more strikingly, the human telomerase reverse transcriptase (TERT) was shown to form a functional RdRP with the RNA component of the mitochondrial RNA processing endoribonuclease (RMRP) (Maida et al, 2009). This enzyme also produces dsRNA from ssRNA templates for Dicer, but is more closely related to reverse transcriptases and viral RdRPs than to cellular RdRPs. In any case, these new findings establish that RdRPs are essential components of all eukaryotic life and suggest novel properties for this class of enzymes.

1.1.2.5 Other components

In addition to the components described above, there are other classes of proteins that are implicated in RNA silencing pathways (see **Figure 1**). During miRNA synthesis, pre-miRNAs are exported from the nucleus to the cytosol using a specific protein called Exportin-5 (Yi et al, 2003). Both RNA and DNA helicases have been shown to be essential for RNA silencing in several organisms (Cogoni & Macino, 1999b; Duchaine et al, 2006; Nakamura et al, 2007), and the role of the RecQ DNA helicase QDE-3 of *Neurospora* will be discussed in detail later (**1.3.2.3**). Before RISC is active, the small duplex siRNA has to be converted into a single-stranded form. An exonuclease known as QIP (QDE-2-interacting protein) has been described in *Neurospora* quelling pathway that specifically cleaves and degrades the passenger strand of the siRNA (Maiti et al, 2007). A similar component called C3PO was recently described in *Drosophila*, although this one is an endoribonuclease (Liu et al, 2009). Many small regulatory RNAs have a single 2'-O-methyl residue at their 3' ends. There are specific methyltransferases to catalyze these modifications, for example, HEN1 methylates miRNAs in *Arabidopsis* and Pimet methylates piRNAs in *Drosophila* (Saito et al, 2007; Yu et al, 2005).

In many organisms, an important level of gene expression regulation occurs through chromatin modifications. Small RNAs have often been found to be involved at this stage as well, and the specific mechanisms have been studied especially in the fission yeast (Moazed, 2009). Heterochromatin is generally regarded as a tightly condensed, gene-poor part of the genome that is replicated late during cell division and characterized by hypoacetylated and hypermethylated histone tails, repetitive DNA sequences and transposons (Grewal & Elgin, 2007). In *S. pombe* assembly, maintenance and gene silencing of heterochromatin requires small

RNAs and associated proteins. A complex known as RITS (RNA-induced initiation of transcriptional gene silencing) interacts with an RNA-directed RNA polymerase complex (RDRC) and Argonaute siRNA chaperone complex (ARC) to silence heterochromatic RNA transcripts (Buker et al, 2007; Motamedi et al, 2004; Verdel et al, 2004). All in all, the various RNA silencing pathways have dozens of RNA and protein components with multiple cellular functions, and several are still presumably waiting for discovery. For example, a genome-wide study in *Neurospora* revealed 60 genes that are activated by dsRNA (Choudhary et al, 2007).

1.1.3 RNA silencing pathways

Now that the RNA and protein components of the various RNA silencing pathways have been described, the details of the pathways will be reviewed below. The most common pathways function through siRNAs, miRNAs and piRNAs (**Figure 1**).

1.1.3.1 siRNA pathway

The siRNA pathway, often called the RNAi pathway begins with the Dicer-directed cleavage of long dsRNA (see **Figure 1A**). As previously mentioned, the trigger dsRNA can originate from transcription of pseudogenes and transposons, convergent or bidirectional transcription of other genes, or be the product of RdRP catalysis (Ghildiyal & Zamore, 2009). In some cases, viral genomes may initiate RNA silencing (Hamilton & Baulcombe, 1999). Whatever the source, the dsRNA is cleaved by Dicer into siRNAs which are RNA duplexes with 5' phosphates and 3' hydroxyls. Each of the ~21 nt strand is base-paired with the other in a way that makes the duplex ~19 nt and leaves 2 nt 3' overhangs (Elbashir et al, 2001a; Elbashir et al, 2001b; Zamore et al, 2000). In *Drosophila*, siRNAs are cleaved by DCR-2 that associates with R2D2 forming the RLC (Liu et al, 2003). A very recent study shows, however, that both Loqs and R2D2 are required for silencing triggered by both endogenous and exogenous dsRNA (Marques et al, 2010). It seems that DCR-2 processes the siRNAs primarily in association with Loqs, and loads them with R2D2 into Ago2-containing RISCs.

The strand that eventually brings the RISC to its mRNA target is called the guide strand while the other one is known as the passenger strand. The passenger strand is usually degraded and the guide strand loaded into the RISC. This is a crucial step in the process of RNAi, as loading of the incorrect strand would lead to adverse cellular responses. It has been shown that the strand of the siRNA duplex that is thermodynamically less stable in its 5' end usually becomes the guide strand, while the one with the more stable 5' end is often degraded (Khvorova et al, 2003; Pei & Tuschl, 2006; Schwarz et al, 2003). R2D2 may be one of the factors that recognize this variation (Tomari et al, 2004). However, chemical modifications seem to exert an effect on strand selection as well, since a single 5'-O-methyl group in a siRNA strand efficiently reduces its incorporation into RISC (Chen et al, 2008). In some cases, both strands of a siRNA can be loaded into RISCs with equal efficiencies (Wei et al, 2009).

A RISC that is associated with a double-stranded siRNA is often called a pre-RISC. The activation of a pre-RISC into a mature, cleavage-competent RISC requires the cleavage and exclusion of the passenger

strand. The 5' phosphate of the guide strand is inserted into a binding pocket close to the interface of the PIWI and MID domains of the Argonaute, while the 3' end binds to the PAZ domain (see **1.1.2.2**). The passenger strand is cleaved by the PIWI domain, which subsequently leads to RISC maturation (Ma et al, 2005; Matranga et al, 2005; Parker et al, 2005; Rivas et al, 2005; Wang et al, 2008). Nucleases such as QIP and C3PO might assist in the activation (Liu et al, 2009; Maiti et al, 2007). The activated RISC finds its targets by complementarity to the guide siRNA and cleaves the mRNA in a similar fashion as the passenger strand (Elbashir et al, 2001b; Martinez & Tuschl,2004; Song et al, 2004). It has been a matter of some debate whether both ends of the guide siRNA are bound to the Argonaute during mRNA cleavage, or whether the 3' end is dislodged from the PAZ domain (Filipowicz, 2005; Tomari & Zamore, 2005). Recent structural work on *Thermus thermophilus* Ago ternary complexes with guide DNA and target RNA seems to support the latter "two-state" model (Wang et al, 2009). The target RNA recognition and cleavage seem to correlate with the accessibility of the mRNA (Ameres et al, 2007).

1.1.3.2 miRNA pathway

Micro RNAs were discovered already in 1993 in *C. elegans*, although their specific functions and mechanisms of action became evident only a decade later (Lee et al, 1993; Wightman et al, 1993). In these early studies the authors described how the expression of the LIN-14 protein, important for larval development, is post-transcriptionally regulated by the gene products of *lin-4*. They showed that *lin-4* does not encode for a protein, but two small RNA transcripts that have complementarities with several elements within the 3' untranslated region (3' UTR) of *lin-14*. They further established that the *lin-14* transcripts are not degraded, but their translation is somehow inhibited. Seven years later, another small RNA called *let-7* was identified in *C. elegans* and shown to have similar regulatory roles (Reinhart et al, 2000). It was also shown that *let-7* sequence is highly conserved and that the RNA is expressed widely in metazoans (Pasquinelli et al, 2000). Shortly thereafter, tens of miRNA genes and miRNAs were described in flies, humans and worms (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee & Ambros, 2001). Today more than 15000 miRNAs from multiple species are listed in the miRBase database (release 16) (Griffiths-Jones et al, 2008).

miRNA biogenesis begins with the transcription of miRNA genes to produce the primary miRNAs which are often polycistronic stem-loop structures with extensive single-stranded areas (see **Figure 1B**). Although there are few comprehensive rules on how to identify a miRNA gene, accurate predictions can be made computationally (Heikkinen et al, 2008; Ohler et al, 2004). Pri-miRNAs are usually transcribed by RNA polymerase II, and are accordingly 5' capped and polyadenylated (Cai et al, 2004; Lee et al, 2004). miRNAs are often located in clusters and many pri-miRNAs are 3 to 4 kb long (Lagos-Quintana et al, 2003; Saini et al, 2007). A subset of miRNAs located within repetitive elements are transcribed by RNA polymerase III (Borchert et al, 2006). Especially in mammals, miRNAs often reside within introns (Kim & Kim, 2007). Sometimes intronic miRNAs, however, may bypass the Microprocessor-directed cleavage altogether, in which case they are known as mirtrons (Okamura et al, 2007; Ruby et al, 2007). Mirtron processing is dependent on intron splicing and independent of Drosha activity.

Pri-miRNAs are processed into pre-miRNAs within the nucleus by the Microprocessor complex containing

Drosha and DGCR8/Pasha (Denli et al, 2004; Gregory et al, 2004; Lee et al, 2003). It has been shown that this occurs co-transcriptionally (Morlando et al, 2008). Within the Microprocessor, DGCR8 functions as a molecular anchor that holds the pri-miRNA in a correct orientation for Drosha processing. The cleavage occurs at a position approximately 11 nt away from the junction of the double-stranded stem and the single-stranded portion of the pri-miRNA (Han et al, 2006). The Microprocessor has been suggested to functionally associate with the spliceosome, further indicating that pri-miRNA processing is co-transcriptional and blurring the distinction between a pre-mRNA and a pri-miRNA (Kataoka et al, 2009). Once processed, the pre-miRNAs are exported to the cytoplasm by Exportin-5 with the help of Ran-GTP (Bohnsack et al, 2004; Yi et al, 2003).

In the cytoplasm, pre-miRNAs are processed into mature miRNAs by Dicer in a similar fashion as siRNAs (Hutvagner et al, 2001). However, as an exception to this rule, the processing of one mouse miRNA (miR-451) has recently been shown to be Dicer-independent and require Ago2 catalysis for maturation (Cheloufi et al, 2010; Cifuentes et al, 2010). The passenger strand in miRNAs is called the miRNA* (miRNA-star), and mature miRNAs are known as miRNA-miRNA* duplexes (Lim et al, 2003). In *Drosophila*, pre-miRNAs are preferentially processed by Dcr-1 in a complex with Loquacious (Forstemann et al, 2005; Jiang et al, 2005; Ye et al, 2007). Once loaded with the miRNA, the activated miRISC finds its targets typically in the 3' UTRs of mRNAs (Bartel, 2009; Doench et al, 2003; Lee et al, 1993; Wightman et al, 1993). Target recognition by miRNAs has been extensively studied by computational and experimental methods, but few general guidelines can be drawn. The 5' nucleotides 2 to 8 of miRNAs is known as the “seed” region, and often this stretch of seven nucleotides is critical for miRNA function (Doench & Sharp, 2004; Lewis et al, 2003). However, also the 3' end of a miRNA is in many cases crucial to stabilize the binding of a miRNA to its mRNA target, and an average miRNA has some 100 potential target sites (Brennecke et al, 2005).

While siRNAs normally guide mRNA degradation through perfect complementarity to their targets, miRNAs are thought to predominantly repress the translation of their target mRNAs (Filipowicz et al, 2008). Translation is often repressed at the initiation or elongation steps. It has been shown that both the 5'-m^7G cap and the 3' poly(A) tail are important for miRNA-mediated translation inhibition (Humphreys et al, 2005). More specifically, miRNAs inhibit the recognition of the m^7G cap by the translation initiation factor eIF4E (cap-binding protein) (Humphreys et al, 2005; Pillai et al, 2005). A domain that binds specifically to the cap has been described in human Ago2, a component of the miRISC (Kiriakidou et al, 2007), suggesting that Ago2 competes with eIF4E for binding to the m^7G cap of target mRNAs. However, this cap-binding motif is absent in the MID domain of *Neurospora* Argonaute (Boland et al, 2010), indicating that this may not be a universal regulation mechanism. On the other hand, miRNAs have also been shown to recruit eIF6 which binds to the ribosomal 60S subunit thereby preventing its binding to the 40S subunit to complete the active 80S ribosome (Chendrimada et al, 2007). Yet other studies report that miRNAs inhibit the elongation step of translation and increase termination or ribosome drop-off (Maroney et al, 2006; Nottrott et al, 2006; Petersen et al, 2006). miRNA-targeted mRNAs as well as Argonaute proteins have been shown to localize into cytoplasmic foci called P-bodies (processing bodies, also known as GW-bodies) that are regarded as sites of mRNA degradation (Liu et al, 2005; Pillai et al, 2005; Sen & Blau, 2005). Consequently, miRNAs have been reported to cause deadenylation and degradation of their target mRNAs (Bagga et al, 2005;

Wu et al, 2006). In a very recent study employing ribosome profiling it was shown that most mammalian miRNAs in fact act to destabilize their target mRNAs (Guo et al, 2010). All in all, the mechanisms by which miRNAs regulate gene expression have not been fully elucidated, but they are likely to occur at several stages of protein synthesis.

The miRNA pathway of plants appears to be slightly different from most other organisms (Jones-Rhoades et al, 2006). As in animals, miRNA genes are usually transcribed by RNA polymerase II (Xie et al, 2005). However, the *Arabidopsis* genome does not contain Drosha homologs and the maturation from a pri-miRNA to pre-miRNA to miRNA seems to be catalyzed by the Dicer-homologue DCL1 in the nucleus (Kurihara & Watanabe, 2004). The miRNA/miRNA* duplexes are methylated by HEN1, and exported to the cytoplasm trough the Exportin-5 homolog HASTY where they associate with AGO1 and find their mRNA targets (Jones-Rhoades et al, 2006; Yu et al, 2005). These activated miRISCs do not usually repress the translation of their targets, but degrade the complementary mRNAs as in the siRNA pathway (Jones-Rhoades et al, 2006; Qi et al, 2005; Tang et al, 2003).

1.1.3.3 piRNA pathway

piRNAs were originally described as a small RNA species regulating the expression levels of repetitive elements in *Drosophila* germline (Aravin et al, 2001). Later they were characterized as an RNA class of 26-31 nt in length that binds exclusively to the Piwi-clade of Argonautes, requires no Dicer cleavage for biosynthesis, and is derived predominantly from only one DNA strand of a genomic piRNA cluster (Aravin et al, 2006; Girard et al, 2006; Grivna et al, 2006; Lau et al, 2006; Vagin et al, 2006). *C. elegans* piRNAs are only 21 nt long and they are known as 21U-RNAs (Batista et al, 2008). piRNA clusters seem to regulate the expression of transposons in the germline. It has been shown that of the *Drosophila* Piwi-clade Argonautes, Piwi and Aubergine (Aub) bind to piRNAs that are antisense to transposon sequences, while Ago3 binds to sense piRNAs (Brennecke et al, 2007; Gunawardane et al, 2007). The piRNAs that bind to Piwi/Aub have a strong bias for a 5' uridine. On the other hand, Ago3-bound piRNAs tend to have an adenine at the tenth position. Moreover, Aub and Ago3 have an *in vitro* slicer activity. These findings have suggested the feed forward or “ping-pong” model of piRNA amplification (Brennecke et al, 2007; Gunawardane et al, 2007): A Piwi/Aub-associated antisense piRNA first binds to a sense transposon mRNA by sequence complementarity (see **Figure 1C**). This mRNA is cleaved at the tenth nucleotide of the piRNA, and loaded from its 5' end to Ago3. The 3' end of the Ago3-bound RNA is processed by an undefined mechanism to yield a sense piRNA, which can in turn guide the cleavage of an antisense transposon transcript. Upon the trimming of the 3' ends of this RNA and its loading to Piwi/Aub, the cycle is completed. However, the mechanisms of primary processing of piRNAs (prior to the amplification loop) are still elusive.

In *Drosophila*, the biological role of piRNAs seems to be mainly in keeping repetitive transposons and other mobile genetic elements under control. While most mammalian piRNA clusters do not match to repetitive elements, they have still been implicated in the control of isolated transposons. Moreover, mammalian piRNAs affect DNA methylation (Aravin et al, 2008; Aravin et al, 2007; Malone & Hannon, 2009). In flies, piRNA content alone is not enough to protect against deleterious effects of transposons, but maternal inheritance patterns of piRNAs have been shown to contribute an epigenetic effect during development

(Brennecke et al, 2008). Recent work has revealed that in addition to the germline piRNAs, there is also a piRNA pathway in somatic cells that uses only Piwi-dependent small RNAs and is independent of the ping-pong amplification cycle (Li et al, 2009; Malone et al, 2009; Saito et al, 2009).

1.1.3.4 Other pathways

The most common RNA silencing pathways are reviewed above. In addition, there are several other variations of these basic mechanisms that are worth mentioning briefly. While all of the previous pathways are examples of post-transcriptional gene silencing, small RNAs have been implicated in transcriptional gene silencing as well. This has been studied extensively in *S. pombe* in the context of chromatin regulation (Moazed, 2009). In the fission yeast, a small RNA pathway is required for the assembly and maintenance of heterochromatin. Evidence suggests that a nascent non-coding RNA transcript is synthesized from centromeric repeats, with which RNA silencing complexes (RITS, RDRC, ARC) associate and guide the methylation of histones. In the ciliated protozoans *Tetrahymena* and *Paramecium*, repetitive DNA is eliminated during sexual development through the function of small RNAs known as scan RNAs (scnRNA) (Malone & Hannon, 2009). Most siRNAs of *Arabidopsis* are dependent on RNA polymerase IV and they have been shown to be uniparentally expressed as a sign of genomic imprinting (Mosher et al, 2009). In *Neurospora*, a population of small RNAs known as qiRNAs are induced by DNA damage (discussed in chapter **4**) (Lee et al, 2009). All in all, novel pathways employing small RNAs are discovered continuously and many of them are closely linked together.

In recent years, the therapeutic use of RNA silencing has become a line of intensive research (Castanotto & Rossi, 2009). Although there are still many unresolved issues such as delivery and off-target effects, many RNAi-based drugs are already in clinical trials. In the future, small RNAs may constitute a major part of pharmacological industry as well as alleviate many animal and plant diseases.

1.2 *Neurospora crassa* as a model organism

1.2.1 History

The first report of *Neurospora crassa* from 1843 describes an orange mould that contaminated bakeries in France. However, it was not until in the early 20th century that the fungus was adopted as a model organism for genetic studies. These were pioneered by Bernard Dodge, who together with Cornelius Shear described the sexual fruiting bodies (perithecia) of *N. crassa* (then called *Monilia sitophila*) and placed it in the genus *Neurospora* together with *N. sitophila* and *N. tetrasperma* (Shear & Dodge, 1927). Dodge went on to describe the mating types *A* and *a*, which segregate in the second meiotic division (Dodge, 1927). By the early 1930s, *Neurospora* had become a powerful tool to study Mendelian genetics, largely because all individual meiotic products could be recovered and used to investigate the segregation of alleles (Davis, 2000; Davis & Perkins, 2002).

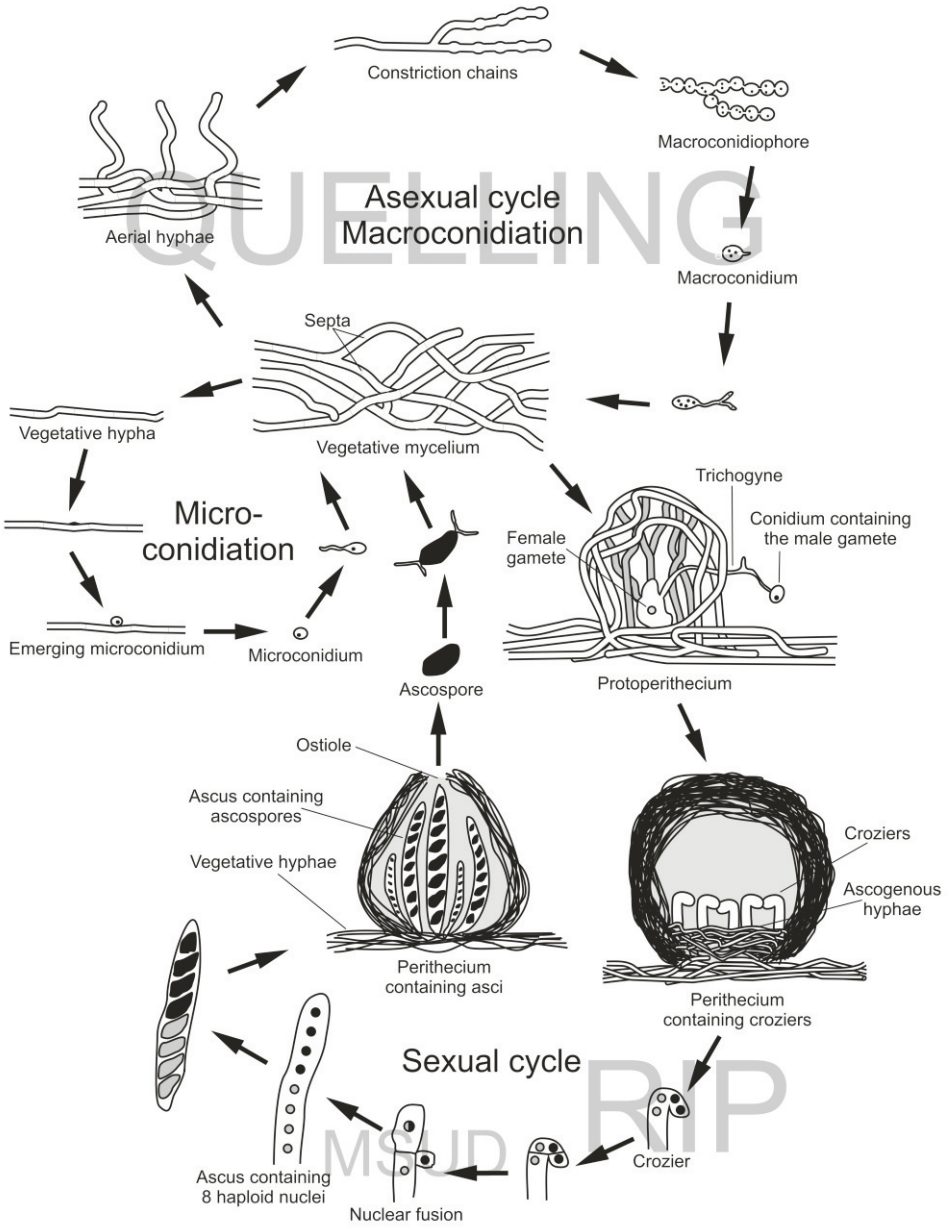
The next major milestone was the "one gene one enzyme" hypothesis by George Beadle and Edward Tatum in 1941, which linked genetics and biochemistry together and was one of the discoveries that led to the era of molecular biology (Beadle & Tatum, 1941). Beadle and Tatum first tried to establish the biochemical basis behind *Drosophila* eye coloration by studying spontaneous mutations. Discouraged by their setbacks with the fly, they turned to *Neurospora* because of its simple growth methods and easily tractable Mendelian genetics. They studied X-ray treated cells and were able to isolate three biochemical *Neurospora* mutants that differed in their nutritional requirements. This is often referred as the turning point that enabled biologists to realize the connection between genes and polypeptides. Together with Joshua Lederberg, who made groundbreaking discoveries in the field of bacterial genetics, Beadle and Tatum were awarded the Nobel Prize in Physiology or Medicine in 1958 (Davis, 2000; Davis & Perkins, 2002).

Escherichia coli and *Saccharomyces cerevisiae* quickly replaced *Neurospora* as the major workhorses of molecular biologists because of their simpler genetics and more elaborate manipulation methods. However, *Neurospora* is still a widely used model organism in the fields of photobiology, circadian rhythms, genome defense, DNA repair, mitochondrial protein import and DNA methylation. Importantly, RNA silencing has been extensively studied in the fungus, and it will be comprehensively discussed in further chapters. Moreover, *Neurospora* serves as a non-pathogenic model to all filamentous fungi, many of which are opportunistic or pathogenic (Borkovich et al, 2004; Davis & Perkins, 2002).

1.2.2 Life cycle and properties

Neurospora has both an asexual and a sexual cycle (**Figure 2**). The asexual cycle is known as macroconidiation and it is characterized by haploid, vegetative hyphae whose filamentous growth habit forms an extensive network called mycelium. Hyphae are called coenocytic as their multiple nuclei are separated by incomplete cell walls (septa) making the mycelium heterokaryotic. When nutrients are exhausted, vegetative hyphae develop into aerial hyphae and macroconidia begin to

Figure 2. Life cycle of *Neurospora crassa*.
A schematic representation of the asexual and sexual cycles of *Neurospora crassa*. The picture was made according to Davis, 2000 and Borkovich et al, 2004. See text for details.



bud on the substrate surface. Macroconidia have one or several nuclei, possess an intensive orange color and are a means of dispersal for the fungus. In addition to macroconidiation, *Neurospora* may also go through another form of asexual cycle known as microconidiation. Microconidia are uninucleate spores that differentiate

from microconidiophores or vegetative hyphae, and germinate with lower efficiency than macroconidia (Borkovich et al, 2004; Davis, 2000).

Nitrogen starvation induces the sexual cycle of *Neurospora*. The cycle begins by vegetative hyphae developing into a female sexual organ called protoperithecium (**Figure 2**). The female gamete is surrounded by the protoperithecium through which emerges a filamentous structure known as a trichogyne. Trichogynes grow towards male gametes (macroconidia) by pheromonic attraction and ultimately a cell fusion occurs. The fusing gametic cells have to be of the opposite mating types (*mat A* or *mat a*). The nuclei divide several times within the perithecium in a mass of ascogenous hyphae. Within specialized structures known as croziers the nuclei undergo premeiotic DNA synthesis and finally fuse into the only diploid stage of *Neurospora* life cycle. The diploid nucleus undergoes two meiotic divisions immediately after fusion, making the developing perithecium into a mature fruiting body. These four meiotic products divide mitotically and develop into eight ascospores. The ascospores are confined within asci, which reside within the beaked fruiting body. The fruiting body shoots mature ascospores to its surroundings through a hole known as the ostiole. Mature ascospores germinate upon heat activation, or remain dormant in the soil for long periods of time (Borkovich et al, 2004; Davis, 2000).

Neurospora has seven chromosomes or linkage groups. The genome was sequenced in 2003 and it is approximately 41 million base pairs (bp) in size containing 10082 predicted protein coding genes with 17118 introns. 41% of the predicted proteins do not match to those of other organisms in the databases, and 57% of *Neurospora* proteins do not show significant similarities to the other two sequenced fungi (*S. cerevisiae* and *S. pombe*) suggesting a divergence early in the evolution. The genome shows a remarkable lack of repetitive sequences, multigene families and gene duplications (Borkovich et al, 2004; Galagan et al, 2003). This is assigned to a phenomenon known as repeat-induced point mutation (RIP) which is a genome defense mechanism found exclusively in fungi. RIP operates during the sexual cycle at the point after the fusion of the gametes but prior to DNA synthesis and nuclear fusion (**Figure 2**). RIP recognizes duplicated sequences that have ~80% nucleotide similarity and introduces C:G to T:A mutations to both of the sequences (Galagan & Selker, 2004). RIPed sequences are also targets of epigenetic gene silencing through DNA methylation (Rountree & Selker, 1997). Within the *Neurospora* genome only the highly repetitive rDNA genes have managed to escape RIP, presumably because they reside within the nucleolus organizer region (NOR) or, as in the case of 5S rDNA genes that are scattered across the genome, are too short for RIP detection. It seems that RIP is an efficient way for fighting against selfish genetic elements, but at the cost of reduced evolutionary potential (Borkovich et al, 2004; Galagan et al, 2003; Galagan & Selker, 2004).

There is another gene silencing mechanism that contributes to the high genomic stability of *Neurospora* known as meiotic silencing of unpaired DNA or MSUD (Shiu et al, 2001). MSUD takes place during the sexual cycle within the diploid nucleus, right after RIP and nuclear fusion but before meiosis and mitosis (**Figure 2**). As the name implies, MSUD recognizes sequences that are not paired during meiosis and silences all homologous DNA. Interestingly, MSUD is dependent on RdRP SAD-1, Argonaute SMS-2 and Dicer DCL-1 (Alexander et al, 2008). Similar components are also required for quelling; the third silencing mechanism of *Neurospora* that is the topic of following chapters.

1.3 Quelling in *Neurospora crassa*

1.3.1 Overview of the pathway

Quelling is an RNA silencing pathway that takes place during the vegetative phase of *Neurospora* lifecycle. First discovered in 1992, it has been an important model for all organisms that possess related silencing phenomena (Romano & Macino, 1992). The following text first gives a general description of quelling, and then provides a comprehensive introduction to the components involved.

The current understanding of the quelling pathway is depicted in **Figure 3** (Fulci & Macino, 2007). Quelling is initiated when repetitive transgenic elements are introduced in *Neurospora*, and it results in the repression of homologous sequences within the genome as well as the transgenes themselves. The repression was shown to be transient and revert to wild-type phenotype as the copy number of the ectopic transgenes decreases (Cogoni et al, 1996; Romano & Macino, 1992). The homologous transgenic element has to be at least 132 bp long and promoter sequences are not competent to induce quelling (Cogoni et al, 1996). Genetic screening revealed *qde* mutants that have impaired quelling (quelling defective) and fall into three complementation groups (Cogoni & Macino, 1997). It was later shown that *qde-1* codes for an RdRP (Cogoni & Macino, 1999a), *qde-2* for an Argonaute (Catalanotto et al, 2000; Catalanotto et al, 2002) and *qde-3* for a DNA helicase of the RecQ family (Cogoni & Macino, 1999b). It is thought that the transgenic element gives rise to an aberrant RNA molecule (aRNA) that is presumably transcribed by RNA polymerase II. QDE-3 has been suggested to either associate with the polymerase or otherwise contribute to the transcription. The aberrant transcript is somehow recognized by QDE-1 and converted to dsRNA. The dsRNA is processed into siRNAs by the two redundant Dicers (DCL-1 and DCL-2) of *Neurospora* (Catalanotto et al, 2004). siRNAs associate with QDE-2 and are converted single-stranded by the combined action of QDE-2 and QIP (Maiti et al, 2007). The active RISC then finds its complementary targets and guides their degradation.

This model is supported by vast experimental evidence, but one of the unresolved issues is the production and character of the aberrant RNA transcript. QDE-1 has been shown to be able to convert ssRNAs into dsRNAs *in vitro* (Makeyev & Bamford, 2002). Moreover, QDE-1 is a rate-limiting factor of quelling *in vivo*, since its over-expression leads to a dramatic increase of silencing efficiency as well as a reduction in the number of transgenes required to induce quelling (Forrest et al, 2004). Also, when dsRNA is directly expressed from a hairpin construct, the requirement for both QDE-1 and QDE-3 is abolished (Catalanotto et al, 2004; Goldoni et al, 2004). However, the features that make a transgenic RNA transcript "aberrant" are completely unknown. This issue is addressed later in this thesis, and a revised model for quelling will be presented.

It is known that in *Neurospora* the RNA silencing pathway does not contribute to histone methylation, as is the case in *S. pombe* (Chicas et al, 2004; Chicas et al, 2005; Moazed, 2009). Although lysine 9 of histone 3 (Lys9H3) is hypermethylated in the transgenic *al-1* loci, the *qde* genes are not required for this to occur (Chicas et al, 2005). Conversely, when the Lys9H3 methylation was relieved (by knocking out the *dim-5* gene encoding for the methyltransferase), the quelled strains reverted more readily back to the wild-type

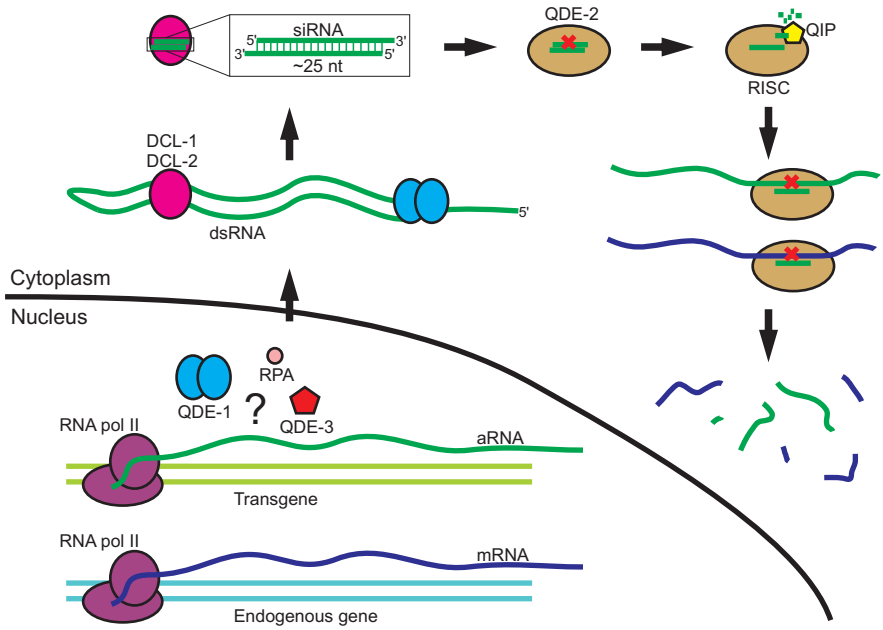


Figure 3. Quelling in *Neurospora crassa*.
A schematic representation of the quelling pathway of *Neurospora*. A canonical mRNA is depicted in blue, and an aberrant RNA in green. The picture was made according to Fulci & Macino, 2007. See text for details.

phenotype. These results indicate that methylation stabilizes the tandem array of the transgenes, but has no effect on the quelling mechanism *per se* (Chicas et al, 2005). However, there seems to be a conceptual correlation between quelling and histone methylation, in the sense that methylated sequences are often relics of RIPed transposons and the quelling pathway produces siRNAs that target these sequences (Chicas et al, 2004). Quelling, RIP and MSUD seem to work in concert to keep transgenic elements under control (Catalanotto et al, 2006; Fulci & Macino, 2007). A striking indication of their efficiency is that no active transposons have been found within the *Neurospora* genome (Galagan et al, 2003). The only known exception is the *Tad* element of an African Adiopodoumé strain. Its expansion is repressed by a pathway that is dependent on QDE-2 and DCL-1/DCL-2 but not on QDE-1 or QDE-3 (Nolan et al, 2005).

In all of the above studies, quelling mechanisms have been addressed mainly by observing phenotypes; i. e. differences in the coloration of mycelia that result from the variable expression levels of the *al-1* gene important for carotenoid biosynthesis. While this is a well-established model for quelling, it could be criticized for being somewhat subjective as it depends on visual inspection. On the other hand, the quelled strains have been subjected to exhaustive molecular analyses that have provided the scientific community with a deep understanding of this "minimal" RNA silencing pathway. Its components are presented next.

1.3.2 Components of the pathway

1.3.2.1 QDE-1

QDE-1 was cloned in 1999 as the first RdRP to be implicated in RNA silencing (Cogoni & Macino, 1999a). Today, it is one of the best-described cellular RdRPs and the only one whose crystal structure has been solved (Salgado et al, 2006). *In vivo*, its main function seems to be in synthesizing dsRNA for Dicer substrate (Catalanotto et al, 2004; Forrest et al, 2004; Goldoni et al, 2004). However, QDE-1 was recently shown to reside both in the nucleus and the cytoplasm of *Neurospora*, and to associate with Replication Protein A (RPA) (Nolan et al, 2008). RPA is a ssDNA binding heterotrimer involved in DNA replication and recombination (Wold, 1997). QDE-1 and RPA are recruited to the transgenic loci, and the accumulation of siRNAs is dependent on ongoing DNA synthesis (Nolan et al, 2008). The results presented in this thesis expand the role of QDE-1 from a dsRNA synthesizing enzyme to a multifunctional RNA polymerase centrally involved in the quelling pathway.

A recombinant QDE-1 and its truncated version QDE-1 ΔN (residues 376-1402 of the wild-type) have brought much insight to the mechanisms and properties of cellular RNA-dependent RNA polymerization (Laurila et al, 2005; Makeyev & Bamford, 2002; Salgado et al, 2006). QDE-1 has been shown to convert heterologous ssRNAs to full-length dsRNAs *in vitro* in a primer-independent (*de novo*) manner. Moreover, it was reported to synthesize small RNAs of 9-21 nts that are scattered along the ssRNA template. Also, the dsRNA synthesis was

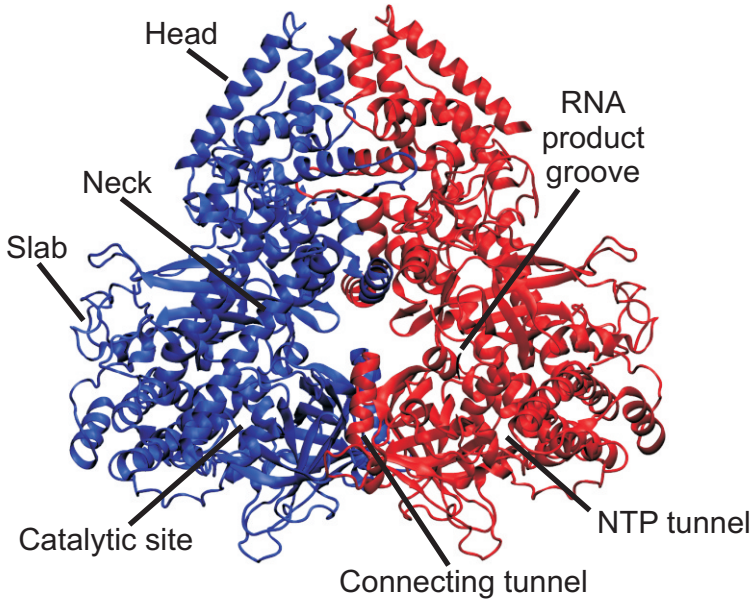


Figure 4. Crystal structure of QDE-1 ΔN.
Crystal structure of QDE-1 ΔN at 2.3 Å resolution (PDB ID: 2J7N). The two monomers of the dimer are shown in blue and red color. The major structural elements are indicated.

shown to be initiated mainly by the folding back of the ssRNA template on itself ("back-priming"). The small 9-21 nt RNAs were suggested to be the main reaction product of RNA synthesis as well as to have a possible biological significance in silencing (Makeyev & Bamford, 2002). Subsequent studies have, however, challenged this view, since quelling can be achieved also by circumventing the requirement for QDE-1 (Catalanotto et al, 2004; Choudhary et al, 2007; Goldoni et al, 2004).

The crystal structure of QDE-1 ΔN has been solved at 2.3 Å resolution (Salgado et al, 2006). The structure revealed two surprising features (**Figure 4**): QDE-1 is a dimeric enzyme, and the fold of its catalytic core is closely similar to those of eukaryotic DNA-dependent RNA polymerases (DdRPs). The subunits are identical to each other, although the disposition of their domains makes the overall conformation more "open" in one of the subunits compared to the more "closed" one in the other. There is an N-terminal "slab" domain in each of the subunits that precedes the catalytic domain. The catalytic domain contains two double-psi β-barrels (DPBB1 and DPBB2) as well as a "flap" domain. The DPBB subdomains superpose nicely with yeast and bacterial DdRPs, but there are no similarities elsewhere in the molecule. The catalytic domain leads to "neck" and "head" domains, which are responsible for most of the intermolecular contacts between the subunits of the dimer. The structure of QDE-1 suggested that the slightly different conformations of the two subunits could represent a "two-stroke motor" where only one of the catalytic sites of the dimer would be active at a given time, possibly reflecting different RNA synthesis initiation modes. The structure, catalytic activities and biological implications of QDE-1 are exhaustively discussed in chapter **4** of this thesis.

1.3.2.2 QDE-2

QDE-2 is the Argonaute of *Neurospora* (Catalanotto et al, 2000). In accordance to its role as a RISC component, it was shown to be essential for quelling but not for siRNA accumulation. Also, siRNAs were observed to co-purify with QDE-2 (Catalanotto et al, 2002). The slicer activity of QDE-2 was confirmed some years later, when it was shown to be essential for both quelling as well as for the generation of single-stranded siRNAs (Maiti et al, 2007). In the same study, it was shown that the maturation of RISC also requires the QIP exonuclease. QIP is essential for RNA silencing and it removes the nicked passenger strand from a siRNA duplex. The crystal structure of QDE-2 MID domain was solved recently (Boland et al, 2010), giving new insights to the cellular roles of Argonautes (discussed in **1.1.2.2**). The presence of dsRNA induces QDE-2 expression and this induction is required for efficient quelling (Choudhary et al, 2007). Moreover, DCL-1 and DCL-2 are important for maintaining the QDE-2 steady-state levels posttranscriptionally. These results indicate that dsRNA (but not siRNAs) is required for induction of *qde-2* mRNA, but QDE-2 protein levels are maintained by Dicers and/or siRNAs. All in all, complex cellular pathways are activated upon dsRNA expression, and there are at least 60 dsRNA-activated genes (DRAGs) in *Neurospora* (Choudhary et al, 2007).

1.3.2.3 QDE-3

The *qde-3* gene codes for a RecQ DNA helicase but is the least understood component of the quelling pathway (Cogoni & Macino, 1999b). It has been shown that QDE-3 is required for siRNA accumulation along with QDE-1, but this requirement is abolished when dsRNA is produced from hairpin constructs (Catalanotto et al, 2002; Catalanotto et al, 2004; Goldoni et al, 2004). The only study that addresses QDE-3 function describes another RecQ DNA helicase of *Neurospora* called RecQ-2 (Pickford et al, 2003). This study shows that RecQ-2 is not required for quelling, but it may function together with QDE-3 in DNA repair. The authors suggested that QDE-3 has a dual role in quelling as well as (together with RecQ-2) in DNA damage control. Interestingly, human QDE-3 homologues WRN and BLM are active in DNA repair and interact with each other (Brosh & Bohr, 2007). Moreover, WRN and BLM interact with RPA which has also been observed for QDE-1 (Nolan et al, 2008). In the quelling pathway, QDE-3 is suggested to detect and resolve aberrant DNA structures in transgenic loci and participate in the transcription of the aberrant RNA transcript (Fulci & Macino, 2007; Nolan et al, 2008; Pickford et al, 2003). A RecQ helicase homologue has also been described to be loosely attached with the rat piRNA complex (Lau et al, 2006). Although no specific function has been assigned to this interaction, it is obvious that *Neurospora* is not the only organism where RecQ helicases are associated with RNA silencing.

1.3.2.4 DCL-1 and DCL-2

Neurospora contains two Dicer homologues: DCL-1 and DCL-2 (Catalanotto et al, 2004). Both proteins are approximately of the same size and are composed of the same domains, apart from a dsRNA binding domain in DCL-2 that is missing in DCL-1. DCL-1 is predicted to be nuclear and DCL-2 cytoplasmic, and both are capable of processing dsRNA into siRNAs *in vitro* and *in vivo*. Importantly, although QDE-2 seems to be responsible for most dsRNA processing, the two proteins are redundant and only a *dcl-1/dcl-2* double mutation impairs siRNA accumulation in *Neurospora*. On the other hand, DCL-1 but not DCL-2 is required for MSUD (see **Figure 2** and chapter **1.2.2**) (Alexander et al, 2008). It is currently not known what are the specific biological functions of the two Dicers.

The components of the quelling pathway are described above. As there are at least 60 DRAGs in *Neurospora*, more genes and proteins likely contribute to the regulation of this complex pathway. In addition, there are some novel small RNA species that are described later in this thesis (chapter **4**).

2 AIMS OF THE STUDY

RNA-dependent RNA polymerases are essential components of many RNA silencing pathways, but their specific reaction mechanisms have received little attention. The recent notion that RdRPs are present in all eukaryotic kingdoms has increased the need to understand these enzymes on a more profound level. In the quelling pathway of *Neurospora crassa* QDE-1 has been shown to be essential for RNA silencing, but its templates and activity modes remain elusive. Although the crystal structure of the dimeric enzyme shows that the catalytic core is related to DdRPs, the specifics of RNA catalysis and indeed the basis for dimerization have not been addressed. Moreover, other components of the quelling pathway are likely to affect the activities of QDE-1, and these interactions are unknown. The specific aims of the present study were:

- to characterize and dissect the *in vitro* activities of recombinant QDE-1 by site-directed mutagenesis

- to further investigate the molecular structure of the dimeric QDE-1 enzyme and assign functions for the different domains

- to bring the *in vitro* findings into a biological context by *in vivo* studies in *Neurospora*

- to work out some of the *in vivo* interaction partners of QDE-1

During the course of this study a novel RNA silencing pathway activated by DNA damage in *Neurospora* was described. This prompted the investigation of the role of QDE-1 in other pathways than quelling.

3 MATERIALS AND METHODS

Plasmids used in this study are listed in **Table 1**. Experimental procedures have been described in detail in the original publications. A summary of methods used is presented in **Table 2**.

Table 1. Plasmids used in this study.

Plasmid name	Description	Reference
pLM659	cDNA-copy of the S-segment of bacteriophage ϕ6 under a T7 promoter	(Gottlieb et al, 1992)
pEM41	Wild-type QDE-1 yeast expression vector with a C-terminal His-tag	(Makeyev & Bamford, 2002)
pEM69	QDE-1 ΔN yeast expression vector with a C-terminal His-tag	(Laurila et al, 2005)
pEM56	QDE-1 ΔN D1011A yeast expression vector	(Makeyev & Bamford, 2002)
pAA8	QDE-1 ΔN D1007A yeast expression vector	**I**
pAA9	QDE-1 ΔN P964A yeast expression vector	**I**
pAA10	QDE-1 ΔN R1091A yeast expression vector	**I**
pAA11	QDE-1 ΔN R738A yeast expression vector	**I**
pAA12	QDE-1 ΔN M1357D yeast expression vector	**I**
pAA13	QDE-1 ΔN M1357C yeast expression vector	**I**
pAA14	QDE-1 ΔN K1119W yeast expression vector	**I**
pAA15	QDE-1 ΔN R944E yeast expression vector	**I**
qa-3FLAG-*qde-1*	Full-length QDE-1 *Neurospora* expression vector containing a FLAG-tag and a *qa-2* promoter	**III**
qa-3FLAG-*rpa-1*	Full-length RPA-1 *Neurospora* expression vector containing a FLAG-tag and a *qa-2* promoter	**III**
c-Myc-His-*qde-1*	Full-length QDE-1 *Neurospora* expression vector containing c-Myc- and His-tags	(He et al, 2005), **II**, **III**
c-Myc-His-*qde-3*	Full-length QDE-3 *Neurospora* expression vector containing c-Myc- and His-tags	**III**
c-Myc-His-*rpa-1*	Full-length RPA-1 *Neurospora* expression vector containing c-Myc- and His-tags	**III**

Table 2. Summary of methods used in this study.

Method	Used in
Standard RNA and DNA techniques	**I, II, III**
ssDNA preparation	**I, III**
Site-directed mutagenesis	**I**
Yeast transformation	**I**
Analytical gel filtration	**I**
Protein expression and purification	**I, II, III**
RNA polymerase activity assays	**I, II, III**
Immunopurification	**II, III**
Nuclease protection assays	**I, II, III**
Nucleic acid analysis by denaturing gel electrophoresis	**I, II, III**
Neurospora crassa culture and manipulation	**II, III**
Small RNA cloning	**II**
qRT-PCR	**II, III**
Quelling assay	**III**
Western blotting	**II, III**
Northern blotting	**II, III**

4 RESULTS AND DISCUSSION

4.1 Site-directed mutagenesis of QDE-1 ΔN (I)

Based on the crystal structure of QDE-1 ΔN (Salgado et al, 2006), several point mutants designed to target critical amino acid residues were created. The mutation sites were chosen to affect each of the domains (**Figures 4** and **5**). Although a catalytically inactive point mutant was already made in a previous study (QDE-1 ΔN^{DA}) (Makeyev & Bamford, 2002), another catalytic aspartate was rendered into alanine (D1007A) to confirm that all the different activities lie within the active site. The incoming template nucleic acid was predicted to be affected by R1091A mutation in the flap domain, and the proposed RNA product tunnel was mutated by converting the R738 into alanine within the DPBB1 subdomain. To tamper with the interaction between the head domains within a QDE-1 dimer, the M1357 was mutated into aspartate to disrupt the helical dimeric interface and into cysteine to lock it together. However, subsequent analytical gel filtrations revealed that all the mutants were dimers (**Table 3**). This confirmed the previous notion of the contact area between the subunits being so extensive (more than 2000 $Å^2$) that QDE-1 behaves as a functional dimer (Salgado et al, 2006). Mutation K1119W was designed to block the predicted nucleotide entrance pore. The rest of the mutations resided within the DPBB2 subdomain: R944E was designed to block the communication tunnel between the two monomers, and P964A lies approximately 11 Å away from the active site.

The above mutants were introduced in *S. cerevisiae*, expressed and purified to near homogeneity. As expected, all the mutant enzymes apart from D1007A were catalytically active. These were used to dissect the different *in vitro* activities of QDE-1. Despite of substantial effort, no new crystal structures could be derived from the point mutants.

Table 3. Analytical gel filtrations of QDE-1 ΔN mutant polymerases.

Protein	Peak elution time (min)*
Apoferritin (Sigma), 443 kDa	113.47
QDE-1 ΔN WT	129.52 (pH 6.3) 127.76 (pH 7.4) 128.03 (pH 8.9)
QDE-1 ΔN D1007A	126.75
QDE-1 ΔN P964A	127.92
QDE-1 ΔN R738A	127.23
QDE-1 ΔN M1357D	127.23
QDE-1 ΔN K1119W	127.23
QDE-1 ΔN R944E	127.12
Alcohol dehydrogenase (Sigma), 150 kDa	137.42 (pH 6.3) 137.31 (pH 7.4) 135.82 (pH 8.9)

* Elution in 50 mM HEPES-KOH pH 7.4, 150 mM NaCl. QDE-1 ΔN WT and alcohol dehydrogenase were also analyzed in 25 mM Bis-Tris pH 6.3, 150 mM NaCl and 50 mM Tris-HCl pH 8.9, 150 mM NaCl.

Figure 5. Mutation sites within QDE-1 sequence.
The figure shows the amino acid sequence of wild-type QDE-1 (accession number EAA29811). The sequence of QDE-1 ΔN is in bold, and the missing N-terminus is overlined in gray. The domains are designated as follows: Slab 390-646; DPBB1 678-792; DPBB2 916-1018; Neck 808-836, 817-913, 1162-1195; Flap 1025-1161; Head 837-888, 1196-1372. Three subdomains of the catalytic domain are outlined by cyan boxes. The mutated amino acids are depicted in red and the mutations are indicated above the residues.

4.2 Characterization of the *in vitro* activities of QDE-1 ΔN (I)

The structure of QDE-1 ΔN showed that its active site resembles the active sites of eukaryotic DNA-dependent RNA polymerases (Salgado et al, 2006). This prompted us to test whether QDE-1 is able to use DNA as its template. Indeed, robust DdRP activity was observed when ssDNA was used as a template. The product of this reaction was sensitive to RQ1 DNase and RNase H, indicating that it was a DNA/RNA hybrid (**II**, **III**). When dsDNA was used, no activity was detected. The different point mutants and varying reaction conditions allowed the characterization and dissection of five *in vitro* activities for the recombinant enzyme: (**i**) RdRP activity, (**ii**) DdRP activity, (**iii**) ssRNA template shift and labeling activity, (**iv**) TNTase activity and (**v**) non-templated ladder activity. The DdRP activity was always considerably higher than the RdRP activity, suggesting that QDE-1 is primarily a DdRP. Moreover, while QDE-1 employs the back-priming mode of RNA synthesis initiation on an ssRNA template, initiation on an ssDNA template does not have this requirement. This differential initiation seems to be one of the mechanisms which allow the polymerase to discriminate between template nucleic acids. All the activities and their variation in the different point mutants are listed in (**I**).

The RNA template shift and labeling activity (**iii**) was previously regarded as the primary activity mode of QDE-1, resulting from the synthesis of small RNAs of 9-21 nt along the template RNA (Makeyev & Bamford, 2002). It was suggested that a subset of these small RNAs would enter the quelling pathway and contribute to RNA silencing. Our results, however, contradict the previous findings (**I**). Many of the point mutants displayed very modest or entirely missing activity (**iii**), and more importantly this activity did not always correlate with efficient RdRP activity (**i**). Our data also suggested that the template shift occurs even with the catalytically inactive enzymes and was partly relieved by treating the reaction products with proteinase K (**III**). It seems likely that QDE-1 binds tightly to its ssRNA template, and the labeling of the shifted template ssRNA is merely a result from TNTase (**iv**) activity. Similarly, the small RNAs of 9-21 nt could just be products of abortative RdRP (**i**) activity (see **4.3**). This conclusion is further supported by several *in vivo* results from *Neurospora* indicating that QDE-1 is dispensable when dsRNA is directly synthesized from hairpin constructs (Catalanotto et al, 2004; Choudhary et al, 2007; Goldoni et al, 2004).

The recombinant QDE-1 also displays two non-templated activities: TNTase (**iv**) and ladder (**v**). TNTase activity is found in various RNA polymerases, and often its biological function is not immediately obvious. This is also the case with QDE-1, although the ability of the P964A mutant to add a non-templated nucleotide on ssRNA but not ssDNA suggests that the activity might be a part of the template recognition mechanism, or indicate back-priming initiation. Activity (**v**) is highly sensitive to reaction conditions, suggesting that it may not be biologically relevant. On the other hand, it occurs only when ATP and UTP are present in the reaction mixture. Moreover, full-length QDE-1 seems to display a more extensive ladder activity than QDE-1 ΔN (unpublished observations), suggesting that it may reflect a still unknown feature of the polymerase.

A surprising result was that the RdRP (**i**) and DdRP (**ii**) activities of QDE-1 are pH-dependent. Activity (**i**) had its optimum at pH 6.3 and this activity decreased as the pH increased. Conversely, activity (**ii**) was modest at a low pH (albeit higher than RdRP activity) and increased as the pH increased, having its optimum at pH 7.4. Also, the ladder activity (**v**) was detected only at low pHs. One can hypothesize that *in*

vivo this pH-dependence reflects different subcellular locations. It has been shown that QDE-1 resides also in the nucleus (Nolan et al, 2008), which may have a higher pH than the cytoplasm (Masuda et al, 1998; Seksek & Bolard, 1996). It would thus seem logical that the DdRP activity would prevail in the nucleus (higher pH) where ssDNA could emerge as a template e.g. during DNA replication. Conversely, the RdRP activity would be the major mode of catalysis in the cytoplasm (lower pH), where ssRNA would be converted to dsRNA. However, QDE-1 activity is surely regulated by many factors other than pH.

4.3 Working model of QDE-1 reaction mechanism (I)

The different catalytic activities, their dependence on reaction conditions and variation among the point mutants allowed us to devise a model of the reaction path for QDE-1 ΔN (**Figure 6**). This model is supported by experimental evidence: 1) RdRP activity requires back-priming while DdRP activity does not, 2) DdRP activity is constantly considerably higher than RdRP activity, 3) the two subunits of a QDE-1 dimer have slightly different conformations (Salgado et al, 2006), 4) the two activities (**i**, **ii**) have different pH optima, 5) the R944E mutant that is predicted to block the communication tunnel is almost devoid of RdRP activity but has a normal level of DdRP activity, 6) The RdRP and DdRP activities do not compete with each other. This model would also explain the ssRNA template shift and labeling activity (**iii**): it results from erroneous activity of the "inactive" catalytic site during the RdRP reaction. It could also be envisioned that the TNTase activity (**iv**) would target an ssRNA template on the other subunit for dsRNA synthesis. This is supported by the notion that the P964A mutant labels only ssRNA but not ssDNA by TNTase activity. The mutated proline lies close to the active site and might convey the templates in proper directions.

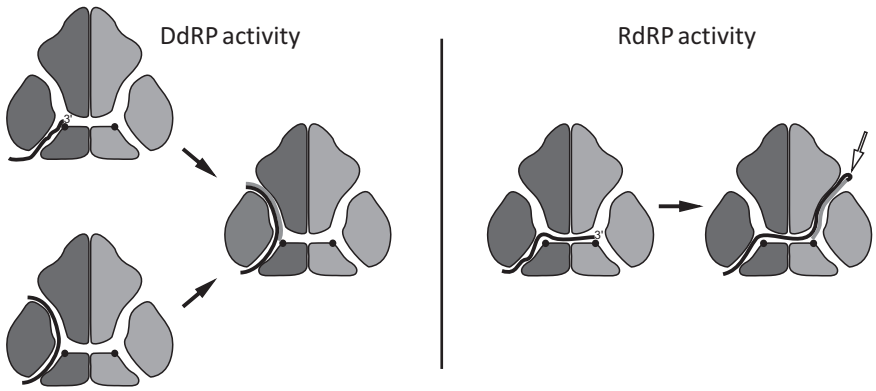


Figure 6. Mechanistic model of QDE-1 catalysis.
The DdRP and RdRP activities employ distinct mechanisms for RNA synthesis initiation. Each monomer of a QDE-1 dimer is colored in different shades of gray, and the active sites are depicted by black circles. Template molecules are depicted in black and newly synthesized RNA in gray. During the DdRP reaction, an ssDNA template is brought to the active site and converted to DNA/RNA hybrid form (left panel). QDE-1 can use both linear (with a 3' end, above) and circular (without a 3' end, below) ssDNA as its template. During the RdRP reaction, an ssRNA template is guided through the communication tunnel to the active site of the adjacent monomer, and converted into dsRNA by the back-priming initiation mode (right panel). The white arrow indicates back-priming initiation on the ssRNA template.

4.4 Cloning and characterization of qiRNAs (II)

In the *Neurospora* quelling pathway, QDE-1 regulates RNA silencing in concert with QDE-2, QDE-3 and the DCLs. Surprisingly, when *Neurospora* cultures were treated with histidine, the expression of QDE-2 increased dramatically. Histidine is known to cause DNA damage in *Neurospora* (Howard & Baker, 1988), and also mutants defective in DNA repair exhibited elevated QDE-2 levels. As QDE-2 is the Argonaute protein of *Neurospora* and contains small RNAs involved in silencing, RNAs associated with QDE-2 after DNA damage were extracted, cloned and sequenced (**II**). Indeed, a population of small RNAs induced after DNA damage was discovered and named QDE-2-interacting small RNAs or qiRNAs. qiRNAs were shorter than *Neurospora* siRNAs, and had a strong bias for 5' uracil (93 %) and 3' adenine (49 %) (**Table 4**). Most qiRNAs matched the ribosomal DNA (rDNA) locus where the nucleolus organizer region is formed by some 200 copies of rDNA repeats (**Table 5**). Although many of the qiRNAs had sequences from mature rRNAs, they also matched to external and internal transcribed spacer regions as well as intergenic spacer regions. These data suggest that qiRNAs are not degradation products of rRNA. The rest of the qiRNAs matched to intergenic regions, open reading frames (ORFs) and tRNAs (**Table 5**). qiRNAs were observed to be derived from long aRNA transcripts, and the biogenesis of qiRNAs was shown to be dependent on QDE-1, QDE-3 and the DCLs. In the *dcl* double mutant the amount of aRNA increased dramatically, suggesting that in the *wt* strain aRNAs are converted into dsRNA and diced into qiRNAs.

Table 4. Sequence bias within qiRNAs.

	U	A	G	C
5' end	93 %	3 %	3 %	1 %
3' end	13 %	49 %	11 %	26 %

Table 5. Genome distribution of qiRNAs.

qiRNA sequence match	% of sequences
rDNA region	86.10
Intergenic region	6.57
ORFs	4.37
tRNA	1.45
No match	1.45

4.5 aRNA synthesis is dependent on QDE-1 (II)

The fact that qiRNAs match to rDNA regions that are not transcribed during conventional rRNA synthesis suggests that aRNAs are transcribed by a non-canonical RNA polymerase. RNA polymerase I is normally responsible for rRNA transcription. However, an RNA polymerase I mutant strain was observed to

have normal amounts of aRNA upon DNA damage induction (**II**). *Neurospora* cultures were also treated with thiolutin that is a potent inhibitor of RNA polymerases I, II and III. Thiolutin had no effect on aRNA production. As QDE-1 has robust DdRP activity, we tested whether it would be responsible for aRNA synthesis. *In vitro*, thiolutin had no effect on RdRP or DdRP activities of QDE-1 (**Figure 7**). This suggested that instead of the canonical RNA polymerases, QDE-1 could function in aRNA transcription. Conversely, the production of aRNAs was completely abolished in a *qde-1* mutant strain. These results strongly suggested that QDE-1 is the DNA-dependent RNA polymerase that synthesizes the aRNAs *in vivo*.

The biological functions of qiRNAs remain somewhat enigmatic. Double-stranded qiRNAs associate with QDE-2 and they are rendered single-stranded to form an active RISC complex. Moreover, the rate of protein synthesis in *Neurospora* is decreased after DNA damage but this reduction is partially relieved in *qde-1* and *qde-3* mutants. It thus seems that qiRNAs are a way for the damaged cell to buy time to repair its DNA, possibly by contributing to DNA damage checkpoints. However, as the decrease in protein synthesis rate is not fully recovered in the quelling mutants, qiRNAs are likely a part of a complex DNA damage regulation pathway. These results show, however, that components of the RNA silencing machinery function in DNA damage response. Indications of this have been reported previously with QDE-3 (Pickford et al, 2003).

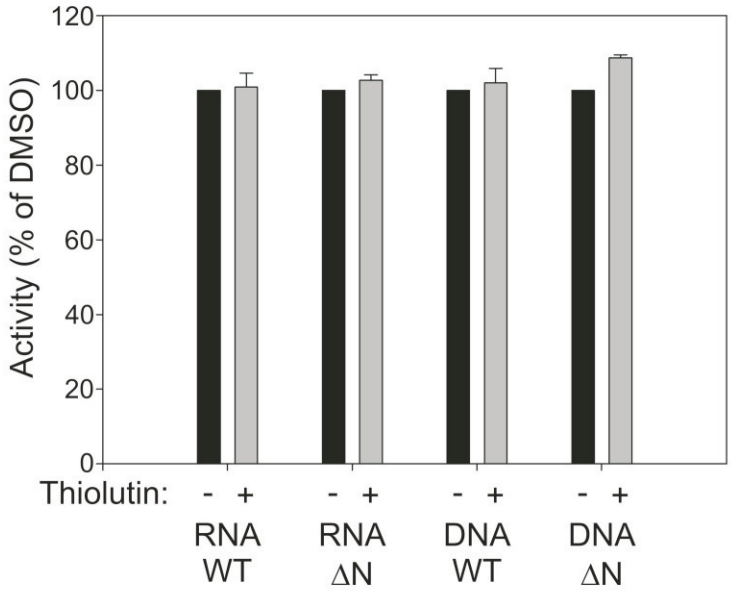


Figure 7. Effect of thiolutin on QDE-1 activity.
Standard QDE-1 reactions were performed with pLM659/SmaI ssRNA or M13mp18 ssDNA as templates, and recombinant QDE-1 WT or QDE-1 ΔN WT as indicated. Thiolutin in dimethyl sulfoxide (DMSO) at 0.4 mg/mL (+) or DMSO only (-) was added in the reactions, and the incorporation of α-^{32}P-UTP in the products (dsRNA and RNA/DNA hybrid, respectively) was quantified by phosphorimaging. The values were normalized to DMSO only levels (=100%). Shown are the averages of three (3) independent experiments. Error bars indicate the standard errors of the mean. The results were similar when thiolutin concentration was increased to 1.0 mg/mL or 4.0 mg/mL.

4.6 The interaction between QDE-1 and RPA is mediated by QDE-3 (III)

Replication protein A (RPA) is a conserved protein complex that is involved in DNA replication and repair (Wold, 1997; Zou et al, 2006). It has been shown previously that RPA interacts with QDE-1 in the nucleus and that the accumulation of siRNAs takes place during DNA replication (Nolan et al, 2008). When DNA damage was induced in *Neurospora rpa* mutants, aRNA and qiRNA expression at the rDNA region was abolished. Moreover, these mutants were also defective in quelling, suggesting that RPA is also an essential component of the quelling pathway. QDE-1 and RPA were observed to directly interact with each other (**III**). However, this interaction was completely abolished in a *qde-3* mutant strain, suggesting that RPA and QDE-3 form a complex that recruits QDE-1 to DNA. Interestingly, mammalian RPA interacts with QDE-3 homologs (BLM and WRN) and stimulates their helicase activity (Brosh & Bohr, 2007). Also, a QDE-3 homolog (rRecQ1) has been described as a component of the rat piRNA complex (Lau et al, 2006). These observations suggest that RPA and RecQ helicases may have functions in the RNA silencing pathways of many organisms.

4.7 QDE-1 and RPA synthesize dsRNA from a ssDNA template *in vitro* (III)

Although it was already previously established that QDE-1 is a *bona fide* DdRP (**I**, **II**), the efficiencies between the RdRP and DdRP reactions had not been compared. When QDE-1 activity assays were conducted with ssRNA or ssDNA with the same length and sequence, the DNA-dependent activity was some 25-fold higher than the RNA-dependent one. The activities of the full-length recombinant QDE-1 were identical to those of QDE-1 ΔN (compare **Figure 8** with Figure 1C of **III**). It was also shown that QDE-1 is able to initiate RNA synthesis on a circular ssDNA template, and that the products of RdRP and DdRP reactions are dsRNA and DNA/RNA hybrid, respectively. Interestingly, we observed that adding recombinant human RPA to QDE-1 DdRP reaction *in vitro* shifts the product from a DNA/RNA hybrid to a dsRNA molecule. This indicates that RPA promotes dsRNA production by either preventing DNA/RNA hybrid formation or by directly regulating QDE-1 activity. The dsRNA synthesized in this fashion was accepted as a Dicer substrate, suggesting that this pathway may function *in vivo*.

4.8 Working model of RNA silencing in *Neurospora* (III)

The data presented in this thesis allow one to suggest a revised model of RNA silencing in *Neurospora crassa* (**Figure 9**). In this model, QDE-1 is the DNA-dependent RNA polymerase that is responsible for aRNA synthesis both during quelling and after DNA damage. QDE-1 is recruited on the transgenic loci or rDNA (upon DNA damage) by RPA and QDE-3. The exact order or composition of this complex is not fully established. After aRNA is synthesized it is transported to the cytoplasm where it is converted into dsRNA by QDE-1. It is unclear whether QDE-1 and/or QDE-3 are transported along with the aRNA molecule into the cytoplasm, or whether separate populations are active in different cellular compartments. The

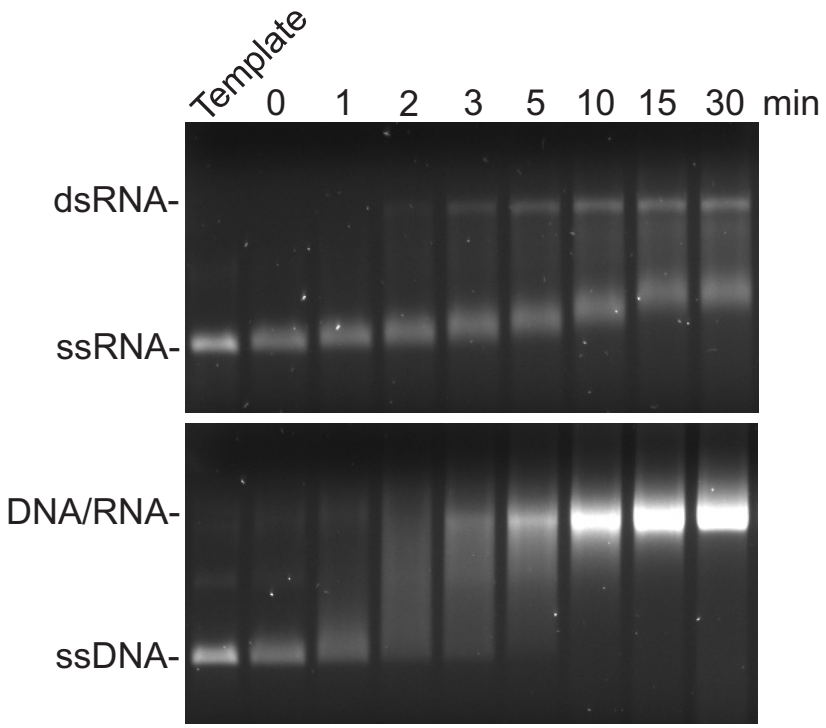


Figure 8. Activitites of full-length, recombinant QDE-1 are identical to those of QDE-1 ΔN.
Standard polymerase activity assays were conducted with full-length recombinant QDE-1 and either ssRNA (upper panel) or ssDNA (lower panel) templates, and samples were taken at indicated time points. Shown are ethidium bromide stained native agarose gels, and positions of templates and products are indicated on the left. See (**III**) for a similar experiment with QDE-1 ΔN.

dsRNA is then cleaved by dicer, which generates siRNAs or qiRNAs. It is not known how the sizes of the small RNAs are determined, but this process probably involves DCL-1, DCL-2 and factors that are yet to be characterized. In any case, siRNAs and qiRNAs associate with QDE-2, form active RISCs and guide the cleavage of transgenic mRNAs or rRNAs.

Recent work has revealed yet other mechanisms of small RNA biogenesis in *Neurospora*. In a comprehensive study analyzing QDE-2-associated small RNAs, pathways that generate miRNA-like small RNAs (milRNAs) and Dicer-independent small interfering RNAs (disiRNAs) were described (Lee et al, 2010). milRNAs are produced by at least four distinct mechanisms that employ both components of the quelling pathway (QDE-2, QIP, DCLs) as well as novel cellular factors. disiRNAs on the other hand seem to be generated by overlapping sense and antisense transcription without the involvement of known quelling components. These results together with the ones presented in this thesis establish that small RNA pathways of *Neurospora* are far more extensive than previously thought. The various regulatory mechanisms described in this filamentous fungus serve as an important reference point as similar systems are unveiled in other eukaryotic organisms.

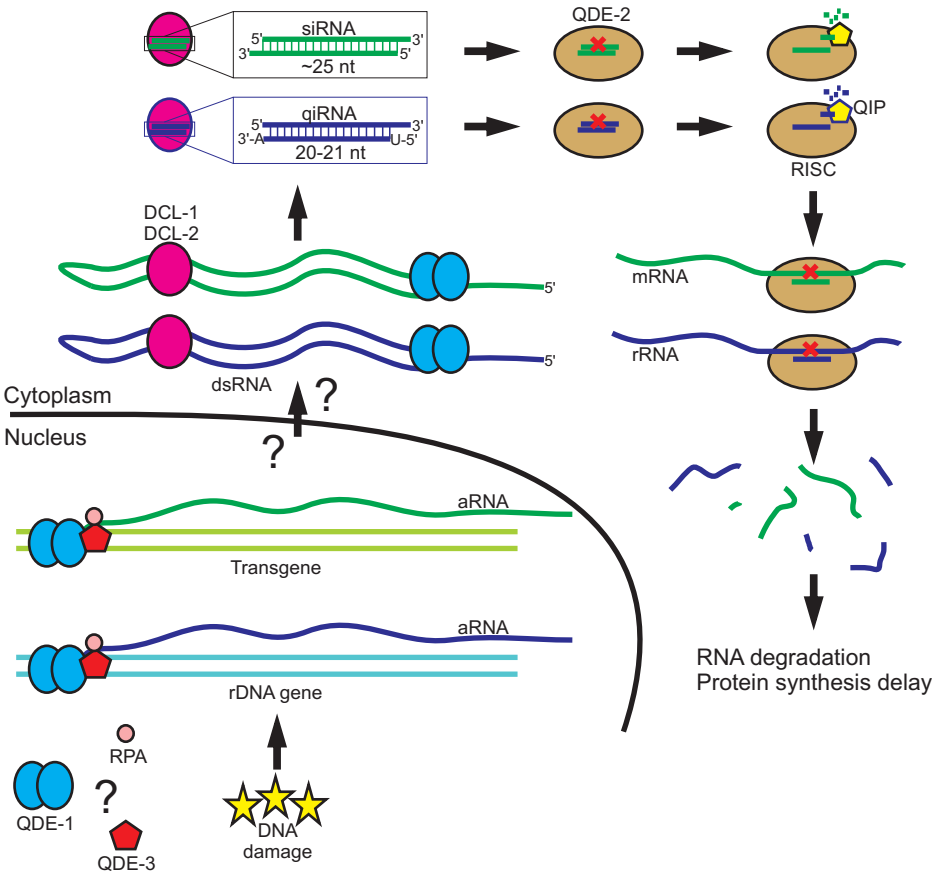


Figure 9. Revisited model of the RNA silencing pathways of *Neurospora*.
A revisited model of the qiRNA and quelling pathways in *Neurospora*. Aberrant RNA synthesized after DNA damage is depicted in blue, and aRNA from a repetitive transgenic element is shown in green. This picture is modified from **Figure 3**. See text for details.

5 CONCLUSIONS AND FUTURE PROSPECTS

In this thesis the structure, biochemical activities and biological functions of QDE-1 from *Neurospora crassa* were investigated. The major finding was that the enzyme that was previously regarded as an RdRP is actually a *bona fide* DdRP with additional RdRP activity. This result brought some light on the puzzling nature of aRNAs: the aberrancy of an RNA does not lie in the molecule itself but in the way it is synthesized in the first place. The finding may have repercussions to the whole field of RNA silencing, since cellular RdRPs have now been discovered in all kingdoms of eukaryotic life. Moreover, a novel class of small RNAs was discovered that combines DNA damage responses to RNA silencing pathways. This is an example of how small RNAs seem to regulate most cellular functions. Finally, based on this work a revisited model of RNA silencing in *Neurospora crassa* emerged that is supported by experimental evidence.

Much still remains to be studied. Although this work suggested many regulatory components for QDE-1, its precise interaction partners are still largely enigmatic. pH is one of the factors that regulate the switch between RdRP and DdRP activities, but there are surely many more. QDE-3 mediates the association between RPA and QDE-1 in the nucleus, but currently there are no data on QDE-1 interaction partners in the cytoplasm. The presence of dsRNA in a cell is known to activate and inhibit the expression of several genes (DRAGs), and these would be the first candidates to investigate. Moreover, practically nothing is known about the functions of the N-terminus of QDE-1. The full-length QDE-1 seems to have the same activities as the ΔN, but it exhibits a more prominent ladder activity (unpublished observations). A crystal structure of the N-terminus would be highly informative to deduce the functions of this large domain. Also, high resolution structures of other quelling components (QDE-2, QDE-3, DCLs) await for determination. Many of the QDE-1 point mutants have been introduced to a *Neurospora qde-1* knock-out strain, and some of the resulting strains seem to display impaired QDE-2 induction upon histidine treatment (data not shown). However, a thorough analysis of these mutants is needed to specify the functional significance of this phenotype.

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